

# Cytology and Nucleic Acid Synthesis of Parthenogenetically Activated Sea Urchin Eggs

INAUGURAL-DISSERTATION

zur

Erlangung der philosophischen Doktorwürde vorgelegt der

Philosophischen Fakultät II

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Marianne von Ledebur-Villiger

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Begutachtet von den Herren

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## CURRICULUM VITAE

Marianne Elisabeth von Ledebur-Villiger, von Sins (AG), geboren in Zug am 11. 6. 1945. In Cham (ZG) besuchte ich 6 Jahre die Primarschule und anschliessend in Zug das Gymnasium, das ich im Frühling 1964 mit der Matura (Typus B) abschloss. Im Herbst desselben Jahres immatrikulierte ich mich an der Philosophischen Fakultät II der Universität Zürich, um Zoologie zu studieren. Ich besuchte Vorlesungen und Kurse der folgenden Dozenten: Akert, Batschelet, Biegert, Burla, Chen, Dreiding, Eugster, Hadorn, Hediger, Hunsperger, Jungen, Kuhn-Schnyder, Ludwig, Markgraf, Nüesch, Rieber, Schnitter, Tardent, Töndury, Viscontini, Wagner, Wanner, Wehner, Weissmann, Ziswiler.

In folgenden Fächern wurde ich geprüft: Paläontologie und Organische Chemie (1. Vordiplom), Botanik, Anthropologie und Anatomie (2. Vordiplom). 1969 erhielt ich das Diplom in Zoologie.

Im Sommer 1971 heiratete ich Thiedhard von Ledebur.

Die vorliegende Dissertation wurde von Herrn Prof. Dr. Pierre Tardent begutachtet und stand unter der Leitung von Herrn Prof. Dr. Gerhard Czihak.



## CYTOLOGY AND NUCLEIC ACID SYNTHESIS OF PARTHENOGENETICALLY ACTIVATED SEA URCHIN EGGS

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### SUMMARY

Unfertilized eggs of *Paracentrotus lividus* activated with thymol and KCl can develop at least until the pluteus stage. Cytological studies showed that development of artificially activated eggs follows one of four different pathways: monaster formation, cytaster formation, haploid mitosis, or intranuclear chromosome replication. Cytospectrophotometric determination of DNA values from blastula nuclei indicated that parthenogenetic blastulae may contain either  $1n$ ,  $2n$  or  $4n$  nuclei or they may be a mosaic regarding the ploidy of their nuclei. DNA synthesis in artificially activated eggs begins about 2.5 times later as compared to fertilized controls; its rate is about half that of fertilized eggs. The rate of RNA synthesis in activated eggs is similar, though somewhat lower, than in fertilized eggs. Parthenogenetically activated and fertilized eggs show a very similar uptake of thymidine and uridine.

One approach to the study of the mechanism of fertilization consists in attempting to imitate the sperm's action by some known agent (parthenogenetic activation). A great amount of work in this field was done with sea urchin eggs. O. & R. Hertwig [28] were the first to discover that unfertilized eggs of *Paracentrotus lividus* formed a membrane when shaken with chloroform. Later, R. Hertwig [29] obtained 2-cell stages after strychnine treatment and Morgan [48] even got 64-cell stages by using NaCl, KCl or  $MgCl_2$ . Loeb [45] was the first to obtain parthenogenetic plutei of *Arbacia punctulata* by means of his famous double treatment (butyric acid and hypertonic sea water). Following these discoveries, several studies on the physiology, cytology and the development of artificially activated sea urchin eggs were published (see [27] for review). Morgan [48, 49] studied the cytology of *Arbacia* eggs after activation with hypertonic NaCl,  $MgCl_2$

or strychnine solutions. Extensive studies were carried out by Wilson [67] on the cytology of eggs from *Toxopneustes variegatus*, treated by Loeb's  $MgCl_2$ -method. In 1910 Hindle [30] published a cytological study of artificial parthenogenesis in *Strongylocentrotus purpuratus* and Paspaleff [53] for his part studied the cytology of artificially activated *Paracentrotus lividus* eggs.

All these investigators concentrated their attention on the first divisions and gave no information regarding the cytology of the later stages of development. They emphasized the origin of centrioles, asters and spindles in the parthenogenetically activated eggs. As most of these investigations were carried out at the beginning of this century we considered it necessary to apply modern methods to the problems of parthenogenesis in sea urchins.

The aim of this study is to give data on the cytology of artificially activated *Para-*

*centrotus lividus* eggs in correlation with DNA and RNA synthesis during the first hours of development. We further studied the cytology and the DNA content of parthenogenetic blastula nuclei in comparison with those of fertilized control blastulae and compared the rate of precursor uptake of artificially activated and fertilized eggs.

## MATERIAL AND METHODS

Sea urchins *Paracentrotus lividus* were collected either in the bay of Naples (Italy) or near Villefranche-sur-mer (France). The eggs were removed from the ovaries and washed several times in sea water collected from the open sea and filtered with charcoal. Sperm suspensions were obtained by diluting 'dry' (=concentrated) sperm removed from the testicles. In order to obtain a high percentage of synchronously developing embryos it proved to be necessary to employ eggs from only one female for each experiment. Developing eggs were incubated in flat dishes in filtered sea water at 20–22°C.

### Parthenogenetic activation

Washed, unfertilized eggs were treated for 30–50 sec in thymol-sea water (2 parts of sea water saturated with thymol + 8 parts sea water, according to [36]). The eggs were then washed several times with sea water and kept for 20 min in sea water. Thereafter they were transferred for 70 min into sea water made hypertonic by addition of KCl. The concentration was varied, but 12 ml of 2.5 N KCl per 100 ml sea water was usually used (0.27 M). After this treatment the eggs were kept in sea water. In some cases eggs were activated by a KCl treatment for 70 min [46]. Either 12, 16 or 20 ml of 2.5 N KCl were added to 100 ml sea water (0.27 M, 0.34 M or 0.42 M). With this treatment no membrane formation occurred; only the hyaline layer was thickened. (According to Ito [37], KCl removes the primary envelope of the activation calyx and consequently no membrane is formed.) Thereafter the eggs were allowed to develop in normal sea water.

In order to prevent sperm contamination, the following precautions were taken: before opening an animal, instruments and animals were washed under tap water. Males and sperm solutions were always kept on a separate table where also fertilization of controls was performed. As fertilized eggs cleave earlier than artificially activated eggs, it was easy to check parthenogenetic cultures for possible sperm contamination at the time of first cleavage of the controls.

### Cytological methods

Living specimens were observed under Nomarski's interference contrast. An 80  $\mu$ m thread previously

dipped into liquid paraffin was circularly arranged on a slide, a drop of sea water containing embryos was placed in the middle and covered by a coverslip. In an oil chamber of this type the development of parthenogenetically activated eggs could be observed from early cleavage until the hatched blastula stage. Samples for cytological examination were taken every 5 to 10 min after fertilization (p.f.) or after activation (p.a.), fixed in ethanol/acetic acid (3:1) for 10 min, stored in 70% ethanol at –20°C and afterwards stained with orcein, carmine or Feulgen reagents. In each sample 300 eggs were examined and their phase in the mitotic cycle was determined. A phase was considered as beginning at the 50% limit and ending at the 50% limit of the following phase, e.g. prophase starts when 50% of the nuclei are in prophase and ends as soon as 50% of the nuclei have reached metaphase.

### Cytospectrophotometry

Healthy-looking blastulae (which, in contrast to abnormal blastulae, swim actively near the surface of the sea water) were pipetted, concentrated by gentle centrifugation and fixed in ethanol/acetic acid (3:1) for 10 min. They were then stored in 70% ethanol at –20°C.

Cytophotometric DNA measurements were made on nuclei of parthenogenetic blastulae as well as on nuclei of fertilized control blastulae from the same batch of eggs.

Hydrolysis was carried out simultaneously for parthenogenetic blastulae and controls in 4 N HCl at 25°C for 90 min in a water bath. The objects were stained in Schiff's reagent for 90 min at room temperature. The reagent was prepared according to Graumann [24] using Merck's basic pararose-aniline. They were washed in 3 changes of SO<sub>2</sub>-water, rinsed in running tap water for 20 to 30 min, dehydrated in ethanol and mounted in benzylbenzoate. Blastulae were squashed on a slide in order to get one layer of nuclei with as little adhering cytoplasm as possible. Without any delay the relative intensity of the Feulgen reaction per single blastula nucleus was measured cytophotometrically by absorption of monochromatic light at 540 nm with the Ultra-Micro-Spectral-Photometer I of Zeiss (scanning measurements). For every group of values the mean ( $\bar{x}$ ), its statistical error ( $s_m$ ) and the standard deviation (S.D.) were calculated.

### Nucleic acid synthesis

Artificially activated or fertilized eggs were continuously incubated in either <sup>3</sup>H-5-thymidine (spec. act. 17.7 Ci/mM), <sup>14</sup>C-thymidine (58  $\mu$ Ci/mM) or <sup>3</sup>H-5-uridine (25 Ci/mM) (The Radiochemical Centre, Amersham). <sup>3</sup>H-5-uridine is a specific precursor of RNA as the conversion to thymidine preparatory to incorporation into DNA involves methylation in the 5-position and therefore results in loss of the label. In order to prevent possible precursor incorporation by developing bacteria, 150 mg each of streptomycin and penicillin were added per litre sea water. At regular intervals equal egg samples were fixed in

5% trichloroacetic acid (TCA) and subsequently washed in 5 changes of 5% TCA in order to remove the free, non-incorporated precursors. The eggs were then transferred into 70% ethanol and stored at  $-20^{\circ}\text{C}$ . Then eggs were either prepared for scintillation counting or radioautography.

For scintillation counting, eggs were processed according to the method of Czihak et al. [12]. Samples were dried in three changes of ethylether (3 times 1 h), thereafter examined for any particles which could falsify the weight and subsequently further dried in the presence of  $\text{P}_2\text{O}_5$ . The weight was determined on a Cahn electrobalance and the eggs were transferred to scintillation vials. As only humid sea urchin eggs are soluble in Packard's Soluene, the eggs were slightly moistened by water condensation (open vials were placed for several hours on a freezing plate). Then 0.5 ml Soluene was added to each vial. After 2 h at  $70^{\circ}\text{C}$  all eggs were dissolved. Thereafter 10 ml of scintillation fluid containing toluene, PPO (5 g/l) and POPOP (0.5 g/l) were added. The radioactivity was measured in a Packard liquid scintillation counter.

The results in cpm/mg dried eggs were referred to either the fertilization or the activation rate. In analogy to the fertilization rate, the activation rate is defined as the percentage of elevated membranes (which are always rather distant from the egg's surface and therefore easily discernible) caused by the thymol treatment.

For radioautography, eggs were dehydrated with alcohols of increasing concentration, cleared in xylene and embedded with Histoplast in gelatin capsules. Sections were cut at  $7\text{ }\mu\text{m}$  and placed on slides coated with chromealaune gelatine. The Histoplast was removed with xylene and the slides were coated with a Kodak AR10 stripping film in the usual way. The exposure times ranged from a few days to several months. The radioautographs were developed with D19b, fixed, washed and stained with eosin and Ehrlich's haematoxylin as described by Simmel [59].

### *Uptake measurements*

The amount of labelled precursor taken up by a known number of eggs was determined by measuring the decrease of radioactivity in the surrounding sea water. Either fertilized or artificially activated eggs were kept suspended in sea water containing labelled precursor. They were allowed to settle for 2 min before samples consisting of 0.1 or 0.2 ml embryo free sea water from the top layer were removed and mixed with 0.4 ml distilled water. After addition of 10 ml of Bray scintillation medium the radioactivity was determined in a Packard liquid scintillation counter.

## RESULTS

### *Development of parthenogenetically activated eggs*

In unfertilized *Paracentrotus* eggs activated with thymol, membrane elevation takes place.

The activated egg has the same appearance as has the fertilized egg (fig. 1,  $a'$ ,  $a$ ). In some cases activated eggs undergo a normal cleavage leading to regular blastulae, gastrulae and plutei (fig. 1,  $b'$ – $e'$ ) which are indistinguishable from the corresponding stages of fertilized eggs (fig. 1,  $b$ – $e$ ). Development of parthenogenetically activated eggs leading to healthy looking and normally behaving blastulae and plutei occurs in 1–40% of the eggs. The frequency differs from egg batch to egg batch and depends on the season in which the eggs are activated. The optimal concentrations of thymol and KCl must therefore be determined each time. In addition, the properties of individual eggs in any one batch must be taken into account, the eggs of one and the same batch reacting differently in the presence of the same concentrations of activating agents.

During a 2-year period the percentage of swimming parthenogenetic blastulae was always high (30–40%) at the beginning of the spawning season (autumn to early winter) and low (1–3%) at the end of the reproductive season (spring). It was also observed that egg batches with an abnormally high percentage of oocytes always yielded good proportions of parthenogenetic blastulae. Thus, the developmental success of parthenogenetically activated eggs seems to depend upon the maturity of the eggs and probably also upon the temperature to which the sea urchins had been exposed. In many cases, however, development following artificial activation leads to anomalies. Mostly the blastomeres are unequal in size or nuclear division takes place without cell division. Cleavage furrows often begin only from one side of the egg and progress slowly. Quite often embryos with 3 blastomeres are found. Also common is the irregular segregation of the cytoplasm leading to cells lacking a nucleus. In most of these embryos develop-

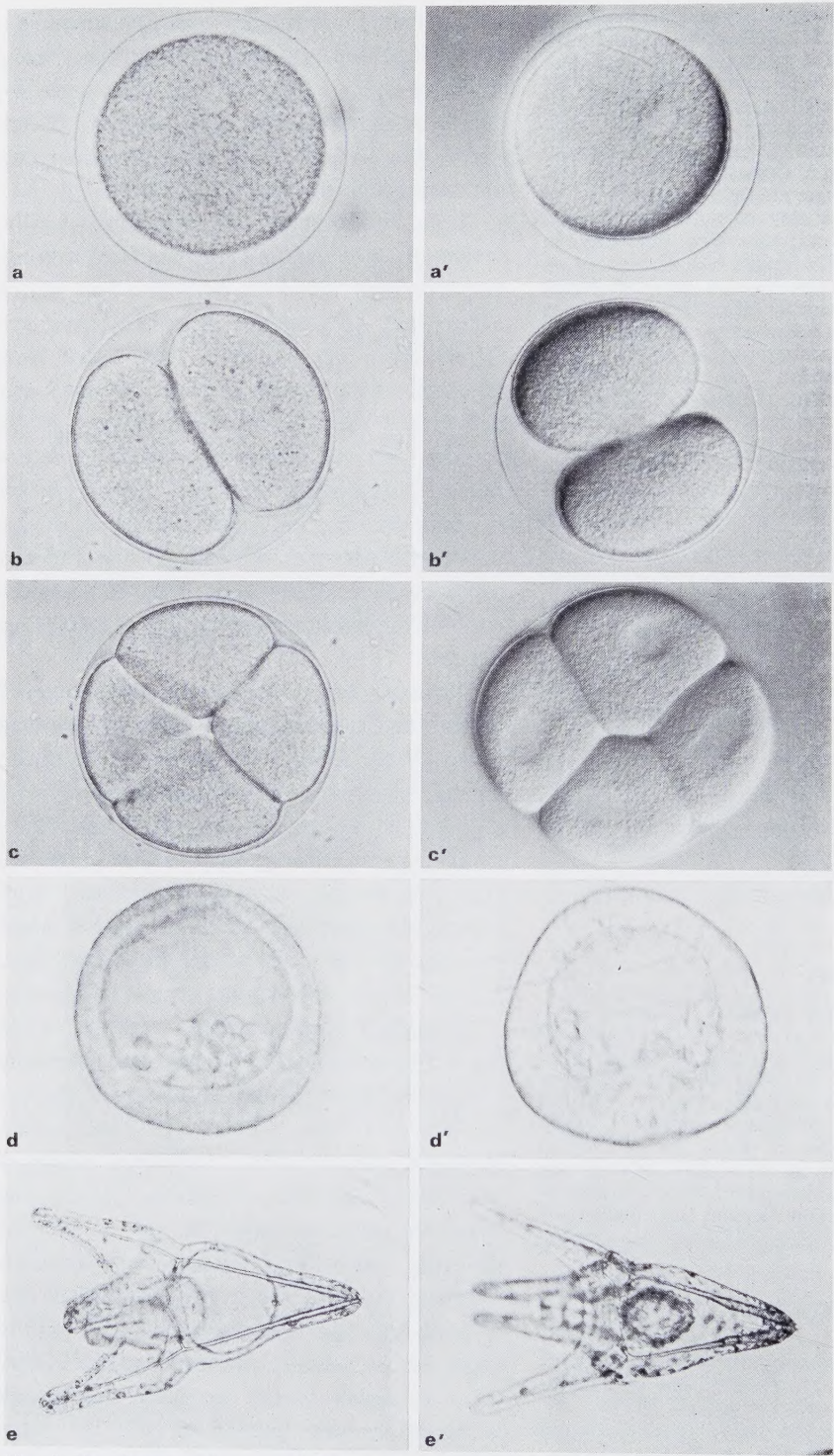


Fig. 1. Developmental stages of *Paracentrotus lividus*. (a-e), Development after fertilization. (a'-e'), development after parthenogenetic activation (thymol-KCl); (a, a') immediately after fertilization or activation respectively; (b, b') 2-cell stage; (c, c') 4-cell stage; (d, d') mesenchyme-blastula; (e, e') pluteus.

ment comes to a standstill. Only a few reach the blastula stage which is characterized by a particularly small blastocoele. These irregular blastulae swim very slowly and are not able to reach the water surface. Many of them do not even hatch and those that do often carry irregular cells attached to their surface. All these abortive blastulae do not gastrulate and stop their development at blastula stage.

Yet even in those cases where a high percentage of parthenogenetically activated eggs develop into plutei, their development is delayed as compared with fertilized eggs (fig. 2). Depending on the batch of eggs parthenogenetic development may be even more retarded: the 2-cell stage may be reached not before 6 h p.a., while the hatching time may vary from 13.5 to 25 h p.a. and the pluteus may be fully developed only 4 days p.a. (dotted lines in fig. 2).

Unfertilized eggs of *Paracentrotus lividus* when allowed to stand in a covered Petri dish for 1–2 days sometimes start cleaving spontaneously, but development never exceeds the 3 to 4-cell stage. In one case we even found eggs which had divided spontaneously inside the ovaries of a normal-looking female. They showed division of plasm and nuclei, but development stopped at the stage of 2 or 3 cells.

#### Cytology of early development

As Feulgen staining failed in the early stages of activated eggs (cf [35] for fertilized eggs), the eggs were stained with orcein or carmine reagents. Immediately after thymol activation the eggs show no visible changes. After being immersed in the KCl-solution, however, the nuclei swell and migrate towards the centre of the egg. About 20 min later small granules adjacent to the nuclear membrane become visible. About 50 min later 18 chromosomes, the haploid set for *Paracentrotus*

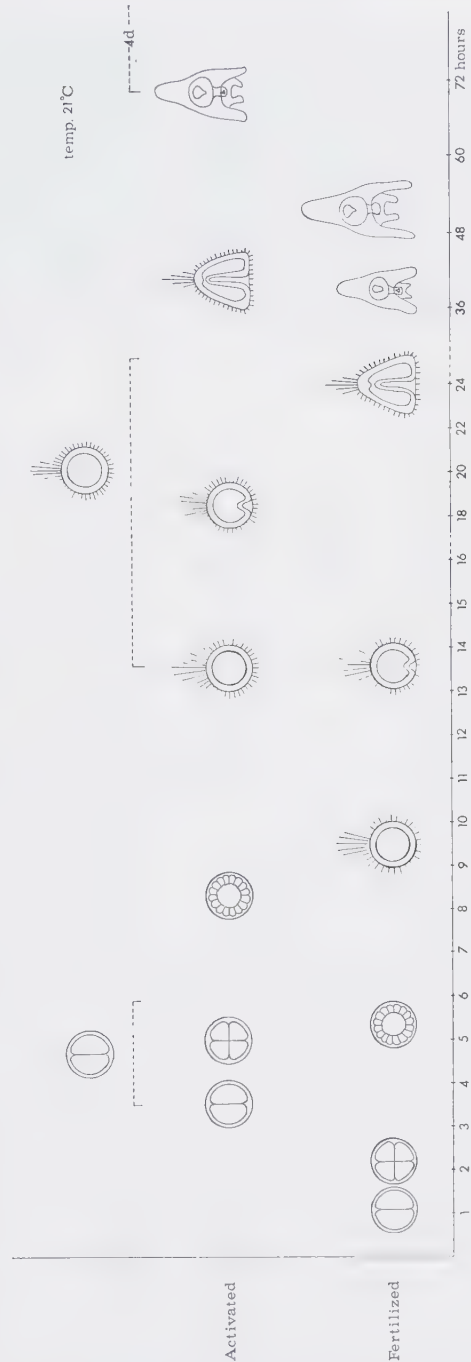


Fig. 2. Chronological comparison of the development of fertilized and that of artificially activated eggs of *Paracentrotus lividus*. For the parthenogenetic development variations in time are indicated by a dotted line.



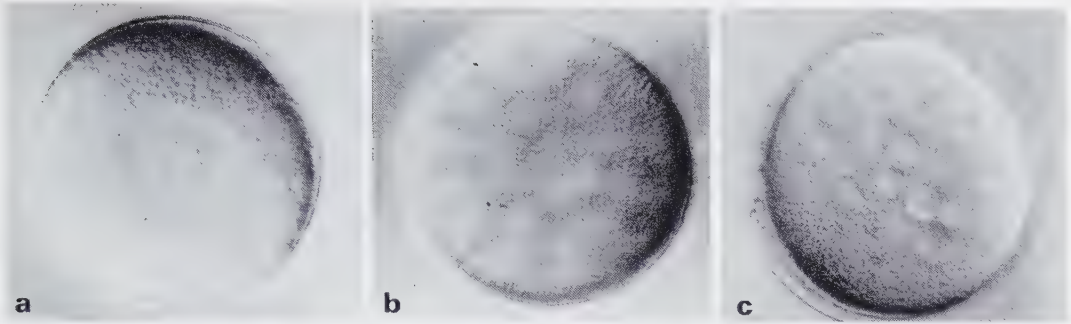


Fig. 4. Parthenogenetically activated eggs under Nomarski's interference contrast. (a) Monaster with chromosomes in circular arrangement; (b) many cytasters in the plasma of an activated egg; (c) multinucleate egg.

*lividus* [5], appear as long thin threads while the nuclear membrane is still intact (prophase). From this stage development can follow four different pathways which are characterized below:

**Monaster:** After disintegration of the nuclear membrane a monaster is formed in or near the centre of the egg. Along its centrifugal rays the chromosomes are moved towards the periphery of the egg, resulting in an irregular circular arrangement of the now strongly spiralized chromosomes (fig. 3a, 4a). At about 2.5 h p.a. the pronounced condensation of the chromosomes is slightly reversed and the chromosomes start dividing (metaphase). About half an hour later the daughter chromosomes, still arranged side by side, are seen separating (anaphase, fig. 3b). At this stage the monaster disappears. The 36 purely maternal chromosomes now enter a telophase-like stage: they become less visible and each pair of chromosomes becomes surrounded by a nuclear vesicle. If these vesicles are distant from each other, the forming karyomeres either do not fuse

at all or only adjacent ones do (fig. 3c). This leads to a varying number of small nuclei each containing a different number of chromosomes. These nuclei may subsequently undergo a few mitotic divisions, in many cases followed by irregular cell divisions, but such embryos soon stop developing. If, however, all the vesicles are closely packed, i.e. if the chromosomes initially are not too distant from one another, they all fuse to form a single diploid nucleus. By subsequent normal mitosis a diploid embryo may arise from such a cell. Not infrequently, however, after an interval of about 1 h, a second monaster is formed (fig. 3d) and once more a replication of the chromosomes takes place. Again the nuclear vesicles fuse either to several small nuclei or, if they are all close together, to a tetraploid nucleus. This  $4n$  nucleus now enters normal mitosis, ending up in a tetraploid blastula.

**Cytaster:** Instead of a monaster several small cytasters appear in the activated cytoplasm (fig. 4b). Those cytasters located near the nucleus take an active part in the distri-

Fig. 3. The cytology of parthenogenetically activated eggs. (a–d) Monaster: (a) first monaster with 18 chromosomes, 2.5 h p.a.,  $\times 1\,280$ ; (b) doubled chromosomes (anaphase), 3 h p.a.,  $\times 1\,800$ ; (c) fusing of chromosomes into karyomeres, 3.5 h p.a.,  $\times 1\,100$ ; (d) second monaster with 36 chromosomes, 4.5 h p.a.,  $\times 1\,200$ ; (e) Cytaster: four-pole mitosis leading to irregular distribution of the 36 chromosomes, 4 h p.a.,  $\times 2\,200$ ; (f) Normal haploid mitosis, 2 h p.a.,  $\times 2\,000$ ; (g–h) intranuclear division of chromosomes: (g) karyogene spindle, 2.5 h p.a.,  $\times 2\,000$ ; (h) tetraploid nucleus resulting from a twofold intranuclear chromosome replication, 3.5 h p.a.,  $\times 1\,500$ .

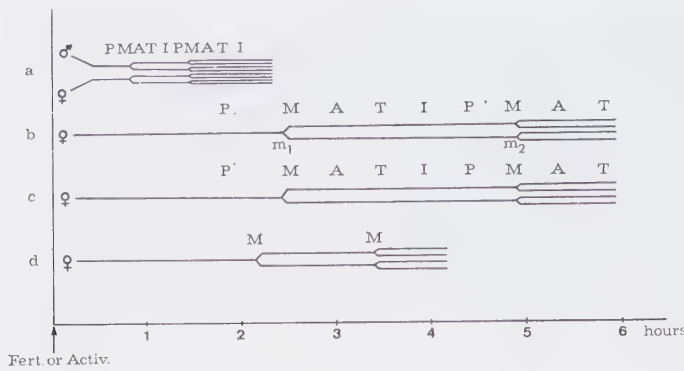


Fig. 5. Comparison of the time necessary for chromosome replication for fertilized (a) and parthenogenetically activated eggs (b-d): (b) Monaster, cytaster; (c) haploid mitosis; (d) intranuclear doubling of chromosomes. P, prophase; M, metaphase; A, anaphase; T, telophase; I, interphase;  $m_1$ , first monaster;  $m_2$ , second monaster.

bution of the chromosomes. The mitotic figures exhibit 3, 4 or even more poles causing irregular distribution of the chromosomes (fig. 3e). This competition for chromosomes can take place after the first prophase or even more often after the doubling of the chromosomes. In both cases the forming nuclei contain varying numbers of chromosomes. These nuclei may divide without division of the cytoplasm resulting in a cell containing up to 30 nuclei (fig. 4c). These nuclear divisions can be followed by cell divisions whereby the larger nuclei are embedded in an accordingly larger portion of cytoplasm than the small nuclei, following a certain nucleo-cytoplasmic relation [8]. These cells of different sizes undergo a few divisions, but development of this type soon stops. A complete haploid set of chromosomes seems to be essential for normal development.

**Haploid mitosis:** In a few cases the activation of the egg results in a normal mitosis involving only the 18 maternal chromosomes (fig. 3f). A normal-looking mitotic apparatus is formed and the chromosomes are doubled and distributed to the two poles forming two haploid daughter nuclei. This mitosis is followed by a cell division. From this 2-cell stage a normal haploid blastula may arise.

**Intranuclear division of chromosomes:** In a few eggs the artificial activation induces the formation of a karyogene spindle. In most

cases this occurs while the nucleus is still excentric. The nucleus swells, its shape becomes elliptical and the nuclear membrane finally disappears. During this process the chromosomes condense, appearing like thick rods. A karyogene spindle whose poles show no astral rays is built up. The chromosomes divide and are pulled somewhat towards the poles of the spindle (fig. 3g). No daughter nuclei are formed. Thereafter the spindle apparatus disappears leaving a diploid nucleus. After varying time intervals this diploid nucleus is able to double its chromosome number by means of the same process. The result is a tetraploid nucleus (fig. 3h). In a few cases this process was observed to be repeated once more leading to an octoploid nucleus. It was not possible to follow the further development of these nuclei as they are indistinguishable from the  $2n$  and  $4n$  nuclei arising from a monaster. Octoploid nuclei were never found in viable blastulae.

Sometimes two of the described developmental pathways may occur in combination. There are for example eggs with one monaster and several cytasters in one blastomere. Monasters with doubling chromosomes were also observed in later stages of development, mostly in only a part of the blastomeres giving rise to mosaic embryos with respect to the chromosomal content of their nuclei.

Different batches of eggs behave differently

as to the frequency of these cytological phenomena. In egg batches collected early in the spawning season (October, November) all four pathways could be observed while late batches of eggs (April, May) showed almost only monasters or intranuclear doubling of chromosomes; no mitotic apparatus was formed and therefore the chromosomes were not distributed into daughter nuclei. This explains why at the end of the reproductional season only very few activated eggs develop into swimming blastulae.

The time necessary for chromosome replication is distinctly longer in parthenogenetically activated eggs than it is in fertilized controls (fig. 5). It is strikingly similar for monaster, cytaster and haploid mitosis of artificially activated eggs. Only the intranuclear doubling of chromosomes is slightly accelerated. The same holds true for each phase of the mitotic cycle which in activated eggs is clearly longer (fig. 5) than in fertilized eggs.

Table 1. *Feulgen values of prophase and metaphase nuclei from parthenogenetic blastulae 24 h p. a. and of metaphase and anaphase nuclei from normal control blastulae 15 h p.f.*

Hydrolysis was in 4 N HCl at 25°C for 90 min

|                                      | <i>n</i> | $\bar{x} \pm s_m$ | S.D. |
|--------------------------------------|----------|-------------------|------|
| <i>Parthenogenetic blastulae</i>     |          |                   |      |
| Prophases and metaphases             |          |                   |      |
| I                                    | 10       | 21.97 + 1.25      | 3.95 |
| II                                   | 40       | 41.15 ± 0.69      | 4.38 |
| III                                  | 70       | 81.01 ± 1.00      | 8.33 |
| Ratio $\bar{x}_{II}/\bar{x}_I$       |          | 1.87              |      |
| Ratio $\bar{x}_{III}/\bar{x}_{II}$   |          | 1.97              |      |
| <i>Normal blastulae</i>              |          |                   |      |
| Metaphases                           | 50       | 33.94 ± 0.53      | 3.75 |
| Anaphases                            | 50       | 16.63 ± 0.24      | 1.71 |
| Ratio $\bar{x}_{meta}/\bar{x}_{ana}$ |          | 2.04              |      |

*n*, number of measured nuclei.

$\bar{x}$ , mean of extinction integrals.

*s<sub>m</sub>*, statistical error of the mean.

I, II, III – values for first, second or third group of parthenogenetic nuclei respectively.

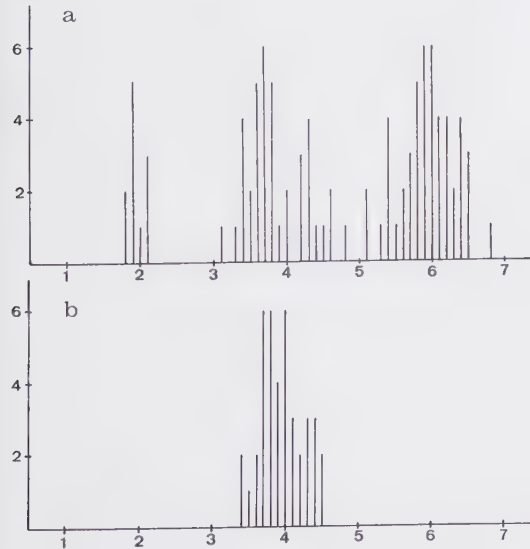


Fig. 6. *Abcissa*: arbitrary units of diameter; *ordinate*: frequency of measured values.

Distribution of diameter measurements of interphase nuclei from blastulae of *Paracentrotus lividus*. (a) parthenogenetic blastulae, 24 h p.a.; (b) normal control blastulae, 15 h p.f.

#### Cytology and DNA content of nuclei from the hatched blastula

In a single blastula either a few large cells containing large nuclei or many small cells containing small nuclei or even cells and nuclei of different sizes were found. Thus the balance between cell size and cell number keeps the size of the embryos remarkably constant.

The measurements of the diameter of the Feulgen-stained interphase nuclei from parthenogenetic blastulae revealed three classes of nuclei (fig. 6a). An analysis of these data indicates that the nuclear diameter of the middle group of activated blastulae corresponds to that of 2*n* nuclei of the controls (fig. 6b) and suggests that the other two classes represent 1*n* and 4*n* nuclei respectively. These findings were confirmed by spectrophotometric determination of the DNA con-



Fig. 7. *Abscissa*: arbitrary units of extinction integrals; *ordinate*: frequency of measured values.

Distribution of Feulgen values from parthenogenetic blastulae, 24 h p.a.: (a) interphase nuclei; (b) prophase and metaphase nuclei; and from normal blastulae, 15 h p.f.; (c) interphase nuclei; (d) metaphase and anaphase nuclei. Hydrolysis was in 4 N HCl at 25°C for 90 min.

tent of Feulgen-stained blastula nuclei. The measurements include prophase and metaphase nuclei, both featuring the replicated amount of DNA, as well as anaphase nuclei, showing the non-replicated DNA values, and interphase nuclei which show a spectrum of DNA values ranging from the non-replicated to the replicated amount of DNA. Values from metaphase and prophase nuclei of parthenogenetic blastulae can be divided into three groups (I, II, III in fig. 7*b*). The mean values of these three groups are terms of a geometric progression, forming ratios of approx. 1:2:4 (table 1). In some favourable cases the chromosomes in metaphase plates of parthenogenetic blastulae could be counted and subsequently their DNA content was measured. Table 2 gives the correlation between the number of chromosomes and their extinction integral. These data confirm that group I in fig. 7*b* represents the DNA content of haploid nuclei, group II the DNA value of diploid nuclei and group III the DNA amount of tetraploid nuclei. As the nuclei were chosen at random it can be concluded that in the examined parthenogenetic blastulae most nuclei exhibit the tetraploid set of chromosomes while nuclei with the haploid set are relatively rare.

For normal control blastulae the DNA values for interphase, metaphase and anaphase nuclei are given in fig. 7*c, d* and in table 1. The ratio between the mean of the metaphase values (replicated amount of DNA) and the mean of the anaphase values (non-replicated amount of DNA) lies very near the theoretically expected value of 2. The fact that the  $2n$  values of the control nuclei are somewhat lower than the  $2n$  values of the parthenogenetic nuclei is probably due to a difference in the optimal hydrolysis time for these two types of nuclei. The rather wide variation both in parthenogenetic and control nuclei

Table 2. *Correlation between the number of chromosomes counted in metaphase plates of nuclei from parthenogenetic blastulae and their extinction integral determined using the UMSP I of Zeiss*

| No. of chromosomes                 | Extinction integral |
|------------------------------------|---------------------|
| 19                                 | 23.8                |
| 18                                 | 20.7                |
| $\bar{x} \pm s_m$ 18.5 $\pm$ 0.5   | 22.25 $\pm$ 1.55    |
| 36                                 | 42.3                |
| 35                                 | 46.4                |
| 36                                 | 40.2                |
| 36                                 | 40.4                |
| 35                                 | 42.7                |
| 34                                 | 40.2                |
| 37                                 | 44.1                |
| 36                                 | 35.4                |
| $\bar{x} \pm s_m$ 35.62 $\pm$ 0.32 | 41.46 $\pm$ 1.16    |
| 72                                 | 76.0                |
| 70                                 | 78.4                |
| 74                                 | 73.5                |
| 72                                 | 69.5                |
| 72                                 | 86.2                |
| 70                                 | 73.8                |
| 71                                 | 84.4                |
| 72                                 | 80.1                |
| 72                                 | 94.0                |
| 71                                 | 73.6                |
| $\bar{x} \pm s_m$ 71.60 $\pm$ 0.37 | 78.95 $\pm$ 2.34    |

For abbreviations see table 1.

can be at least partly explained by a different amount of cytoplasm adhering to the nuclei which may interfere with absorption in the spectrophotometric measurements.

There are different types of parthenogenetic blastulae: the non-mosaic type containing nuclei of only either the  $1n$ , the  $2n$  or the  $4n$  value and the mosaic type featuring nuclei of either  $1n$  and  $2n$  or  $2n$  and  $4n$  values. Blastulae with all three levels of ploidy ( $1n$ ,  $2n$ ,  $4n$ ) are an exception. The ratio of the two types of nuclei in a mosaic blastula is not constant; it may vary from 1:1 to 1:9, indicating that the mosaic type can arise during different stages of early development. The distribution of the six types of blastulae found in one

Table 3. Frequency of the 6 different types of parthenogenetic blastulae as found in one batch of activated eggs

|            | Non-mosaic types |           |           | Mosaic types |              |                |
|------------|------------------|-----------|-----------|--------------|--------------|----------------|
| Ploidy     | <i>n</i>         | <i>2n</i> | <i>4n</i> | <i>n/2n</i>  | <i>2n/4n</i> | <i>n/2n/4n</i> |
| Frequency  | 4                | 18        | 42        | 7            | 10           | 1              |
| Percentage | 5 %              | 22 %      | 51 %      | 9 %          | 12 %         | 1 %            |

batch of activated eggs is shown in table 3. Most of the blastulae (78 %) are non-mosaic whereof the tetraploid blastulae form the major part.

Nucleic acid synthesis

DNA synthesis

DNA synthesis in artificially activated eggs was studied in five experiments of <sup>3</sup>H-5-thymidine incorporation by scintillation counting combined with radioautographic and cytological studies.

*Continuous short-term incubation* (1.5 h): Eggs were incubated with <sup>3</sup>H-5-thymidine (spec. act. 17.7 Ci/mM; concentration 7.7 μCi/ml) at 21°C. Labelled thymidine was added immediately after fertilization or activation with thymol respectively. Twenty min p.a. the eggs were transferred from the sea water containing <sup>3</sup>H-thymidine into a KCl solution to which an identical amount of <sup>3</sup>H-TdR had been added. In this medium the eggs remained until the end of the experiment. One half of each sample, taken every 10 min, was examined in a liquid scintillation counter, while the other half was processed for radioautography.

Fertilized eggs show a marked synthetic activity between 10 and 30 min p.f. which is followed by a plateau extending from 30 to 50 min (fig. 8). In comparison, parthenogenetically activated eggs exhibit a very low rate of DNA synthesis. In each sample the per-

centage of labelled nuclei (labelling index) was determined by means of radioautography. Each value is based on the evaluation of 100 randomly chosen nuclei. As the sections were cut at 7 μm, one nucleus may have been assayed twice or three times. The results are summarized in table 4. In fertilized eggs the male and female pronuclei prior to karyogamy were not labelled, even when sections were exposed for more than 4 months. Only after the fusion of the pronuclei at 15 to 20 min p.f. was the synkaryon labelled. The percentage of labelled nuclei remains constant and is close to the percentage of eggs actually fertilized, indicating that each fertilized egg incorporated thymidine. Therefore, for fertilized eggs the values obtained from the scintillation counting need not be corrected. The only exceptions are the 5 min

Table 4. Determination of the percentage of labelled nuclei (labelling index) by radioautography

Fertilized and activated eggs from the short-term incubation experiment (fig. 8). Each value is based on the evaluation of 100 randomly chosen nuclei

| Time p.f. (min)                    | Labelling index (%) | Correction factor |
|------------------------------------|---------------------|-------------------|
| <i>Fertilized eggs</i>             |                     |                   |
| 5                                  | 0                   | —                 |
| 10                                 | 0                   | —                 |
| 15                                 | 12.5                | —                 |
| 20                                 | 62.2                | 1.61              |
| 25                                 | 67.1                | 1.49              |
| 30                                 | 68.0                | 1.47              |
| 35                                 | 64.5                | 1.55              |
| 40                                 | 66.6                | 1.50              |
| 45                                 | 63.7                | 1.57              |
| 50                                 | 64.4                | 1.55              |
| <i>Artificially activated eggs</i> |                     |                   |
| 10-30                              | 0                   | —                 |
| 40                                 | 18.9                | —                 |
| 50                                 | 22.5                | 4.44              |
| 60                                 | 37.0                | 2.70              |
| 70                                 | 37.4                | 2.67              |
| 80                                 | 46.3                | 2.16              |
| 90                                 | 60.7                | 1.65              |

and 10 min values where the radioautographs showed no incorporated thymidine. This difference between scintillation and radioautographic results could be due to the fact that labelled thymidine had just been taken up by the cells from which, in contrast to sections, it could not be entirely washed out. Another possible explanation is that the newly synthesized still small strands of DNA (which according to [3] are sensitive to TCA treatment) are more easily lost from sections than from whole fixed eggs. The dotted line in fig. 8 shows the incorporation curve with values corrected by the radioautographs.

In parthenogenetically activated eggs, however, labelled nuclei could not be detected before 40 min p.a. The label above the nuclei is ringshaped, indicating that at the beginning of DNA synthesis the concentration of labelled precursor is distinctly higher near the nuclear membrane. Later on, the label becomes uniform. The labelling index increases with time. At 90 min p.a. 60% of the nuclei are labelled. This means, as the activation rate of this experiment was 87%, that up to 90 min p.a. only about two-thirds of the eggs which had shown membrane elevation have incorporated thymidine. Therefore the scintillation values must be corrected by referring them to the percentage of labelled nuclei determined radioautographically. The correction factor is:  $1/\text{labelling index}$ . The incorporation curve with values corrected in this way (fig. 8, dotted line) now shows a distinct synthetic phase beginning at 30 min p.a.

**Continuous long-term incubation (6 h):** Eggs were continuously incubated with  $^3\text{H}$ -5-thymidine (17.7 Ci/mM;  $3.4 \mu\text{Ci/ml}$ ) at  $20^\circ\text{C}$ .  $^3\text{H}$ -thymidine was added immediately after fertilization, but for parthenogenetic eggs only at the beginning of the KCl treatment. At 70 min p.a. the activated eggs were transferred into normal sea water containing

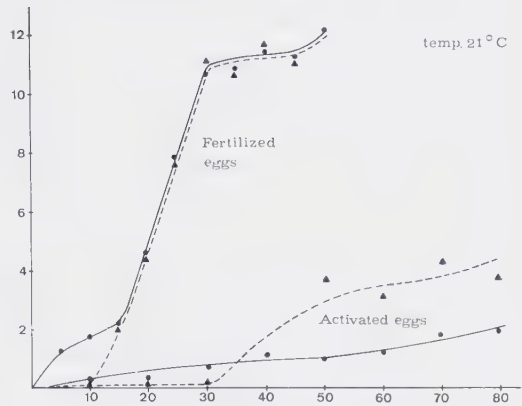


Fig. 8. Abscissa: min; ordinate:  $\times 10^3$  cpm/mg eggs.

Fertilized or activated eggs were continuously incubated with  $^3\text{H}$ -5-thymidine ( $7.7 \mu\text{Ci/ml}$ ,  $17.7 \text{ Ci/mM}$ ). —, cpm/mg dried eggs as determined by liquid scintillation; ----, the same values corrected for the percentage of labelled nuclei as determined in radioautographs (cf table 4).

the same concentration of  $^3\text{H}$ -TdR as the KCl solution had at the beginning.

For the fertilized eggs (upper left part of fig. 9) the percentage of 2- and 4-cell stages respectively (a) as well as the number of chromosomal sets (b) were determined. The first chromosome replication takes place at about 1 h p.f., the second one at about 2 h p.f. The cytological results are summarized in fig. 9c.

The cytological study of 500 artificially activated eggs has shown that 80% of the eggs developed by forming a monaster, 8% by intranuclear doubling of the chromosomes, 2% by forming cytasters while development did not start in 10% of the eggs. In the summary of the cytological results for activated eggs (fig. 9, bottom) only the eggs showing monasters are considered. In activated eggs both doubling of the chromosomes (b) and the sequence of the mitotic phases (c) are much slower than in fertilized eggs. Anaphase and telophase showed strong asynchrony, and only a few nuclei having reached the second prophase and metaphase could be found.

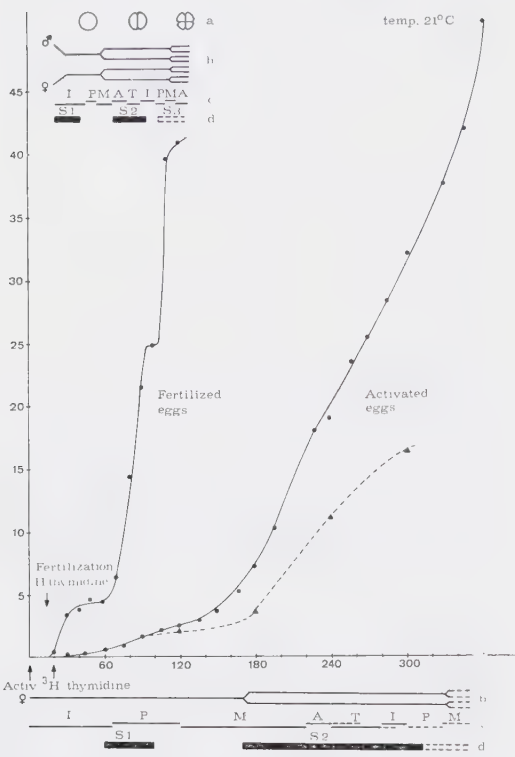


Fig. 9. Abscissa: min; ordinate:  $\times 10^3$  cpm. Fertilized or activated eggs were continuously incubated with  $^3\text{H}$ -5-thymidine (3.4  $\mu\text{Ci/ml}$ , 17.7 Ci/mM) and cpm/mg dried eggs were determined by liquid scintillation (—). These values were corrected for the percentage of labelled nuclei as determined in radioautographs (---). A summary of the cytological studies is given for fertilized eggs in the upper left part of the graph and for artificially activated eggs at the bottom of the graph. (a) stage of eggs; (b) number of chromosomal sets; (c) time and duration of each mitotic phase, I, interphase; P, prophase, M, metaphase, A, anaphase, T, telophase; (d) time and duration of DNA synthesis, S<sub>1</sub>, first; S<sub>2</sub>, second; S<sub>3</sub>, third synthetic phase.

The results of the scintillation counting (fig. 9) also show distinct differences between fertilized and activated eggs: the fertilized eggs have a first synthetic phase from 20 to 40 min, followed by a plateau lasting from 40 to 60 min p.f. Then the second S phase begins. Because of a high asynchrony in the development of these eggs the second plateau is scarcely discernible. The results of the ra-

dioautographic studies for fertilized eggs do not alter the incorporation curve resulting from scintillation counting.

In parthenogenetically activated eggs, however, there is a low rate of DNA synthesis until 140 min p.a., followed by a marked increase. In contrast to those of fertilized eggs the results of the radioautographic studies for activated eggs (table 5) alter distinctly the incorporation curve which results from scintillation counting. The proportion of labelled nuclei increases with time. The incorporation curve for parthenogenetically activated eggs, corrected for the percentage of labelled nuclei, shows now a marked plateau from 90 to 170 min p.a. The values of this plateau are half those of the first plateau for fertilized eggs. If  $^3\text{H}$ -TdR incorporation is a reliable measure of DNA synthesis, this means that the parthenogenetically activated eggs (containing the haploid number of chromosomes) synthesize half the amount of DNA synthesized by the fertilized eggs. After this plateau DNA synthesis shows a continuous increase which is probably due to increasing asynchrony with further development.

A comparison between the cytological data and the results of the scintillation counting reveals that the first S phase of fertilized

Table 5. Determination of the percentage of labelled nuclei (labelling index) by radioautography

Artificially activated eggs from the longtime incubation experiment (fig. 9). Each value is based on the evaluation of 100 randomly chosen nuclei

| Time p.a.<br>(min) | Labelling index<br>% | Correction<br>factor |
|--------------------|----------------------|----------------------|
| 60                 | 36                   | 2.77                 |
| 120                | 55                   | 1.82                 |
| 180                | 68                   | 1.47                 |
| 240                | 63                   | 1.60                 |
| 300                | 80                   | 1.25                 |

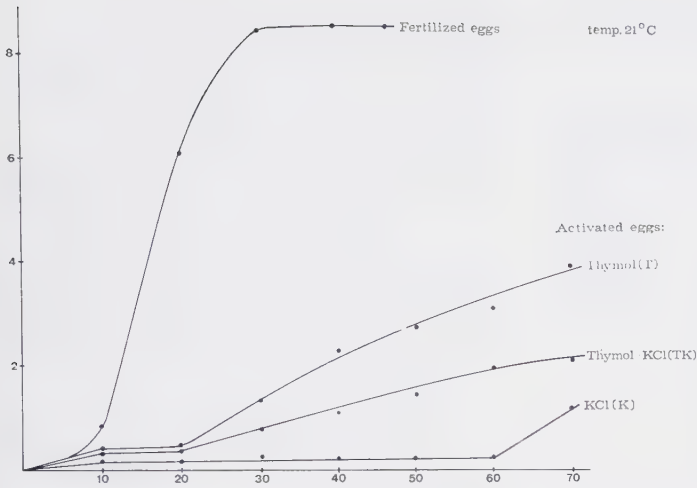


Fig. 10. Abscissa: min; ordinate:  $\times 10^3$  cpm/mg eggs.

Eggs of *Paracentrotus lividus* fertilized or activated either with thymol alone, with thymol-KCl or with KCl alone were continuously incubated with  $^3\text{H}$ -5-thymidine (6.4  $\mu\text{Ci/ml}$ , 17.7 Ci/mM) and cpm/mg dried eggs were determined.

eggs corresponds to the time between fusion of the pronuclei and prophase of the first cleavage, whereas the second synthetic phase coincides with anaphase and telophase of the first cleavage.

Similarly in activated eggs the first S phase corresponds to the first interphase and early prophase (fig. 9c, d). The second synthetic phase coincides with the doubling of the chromosomes in the monaster, but cannot be timed exactly, as further development is too asynchronous.

#### Effect of each activating agent upon the rate of incorporation

Eggs of one female were divided into four equal parts. The concentration of all  $^3\text{H}$ -TdR solutions in this experiment was 6.4  $\mu\text{Ci/ml}$ , the specific activity 17.7 Ci/mM. One-fourth was fertilized and continuously incubated with  $^3\text{H}$ -5-thymidine. The second portion of eggs was activated by thymol alone and transferred immediately thereafter into  $^3\text{H}$ -TdR containing sea water. The third part was activated by thymol and KCl, i.e. after activation with thymol the eggs were exposed for 20 min to radioactive sea water

and thereafter transferred to a radioactive KCl solution. The last portion finally was activated with KCl only, i.e. incubated with a radioactive KCl solution.

Again the fertilized eggs show a first S phase from 10 to 30 min p.f. followed by a plateau (fig. 10). Among the parthenogenetically activated eggs the portion activated by thymol alone shows the highest incorporation rate. The eggs activated with KCl alone start incorporation only at 70 min p.a. Comparison of the thymol and the thymol-KCl curves indicates that KCl given at 20 min p.a. diminishes the incorporation rate of  $^3\text{H}$ -TdR. As will be shown later this effect is due to only a small extent to a diminution of  $^3\text{H}$ -TdR uptake by KCl. It therefore seems possible that KCl has an influence on the incorporation of thymidine into DNA.

Autoradiographic studies of parthenogenetically activated eggs (table 6) demonstrate that in those two portions of eggs where thymol activation was involved almost 100% of the nuclei were labelled at 20 to 30 min p.a. In KCl-activated eggs, however, only after 70 min p.a. do some labelled nuclei appear. Thus in this experiment no correction of the incorporation curves was necessary.

Table 6. Determination of the percentage of labelled nuclei (=labelling index) by radioautography

wggs (from the experiment represented in fig. 10) were either activated by thymol alone (T), by thymol and KCl (TK) or by KCl alone (K). Each value is based on the evaluation of 100 randomly chosen nuclei.

| Time p.a.<br>(min) | Labelling index<br>(%) | Correction<br>factor |
|--------------------|------------------------|----------------------|
| T 10               | 2                      | —                    |
| T 40               | 96                     | 1.04                 |
| T 70               | 97                     | 1.03                 |
| TK 10              | 6                      | —                    |
| TK 40              | 94                     | 1.06                 |
| TK 70              | 95                     | 1.05                 |
| K 10               | 0                      | —                    |
| K 40               | 2                      | —                    |
| K 70               | 14                     | —                    |

### RNA synthesis

RNA synthesis and its correlation with DNA synthesis was studied in two experiments in which fertilized and thymol-KCl activated eggs were exposed simultaneously to  $^3\text{H}$ -5-uridine (25 Ci/mM; 2.88  $\mu\text{Ci/ml}$ ) and  $^{14}\text{C}$ -thymidine (58  $\mu\text{Ci/mM}$ ; 0.12  $\mu\text{Ci/ml}$ ) at 22°C. The values of the scintillation counting which are corrected for either a fertilization rate of 97 % or an activation rate of 75 % are given in fig. 11.

In the fertilized eggs the first, second, third and the beginning of the fourth step of active DNA synthesis are clearly visible. RNA synthesis on the other hand, (see separate scale at the right hand side of the graph) remains at a low level for about 2 h 40 min p.f. From this point onwards there is a marked increase in  $^3\text{H}$ -UdR incorporation, the beginning of which coincides with the fourth synthetic phase of DNA synthesis and corresponds to the division leading from the 4-cell stage to the 8-cell stage. These findings suggest that RNA synthesis already starts

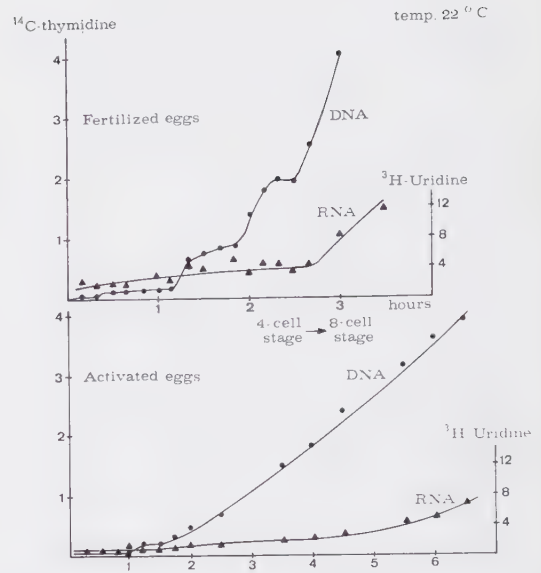


Fig. 11. Abscissa: hours; ordinate:  $\times 10^3$  cpm/mg eggs. Eggs either fertilized or activated by thymol-KCl treatment were continuously incubated in sea water which was doubly labelled with  $^3\text{H}$ -5-uridine (2.88  $\mu\text{Ci/ml}$ , 25 Ci/mM) and  $^{14}\text{C}$ -TdR (0.12  $\mu\text{Ci/ml}$ , 58  $\mu\text{Ci/mM}$ ). The incorporation rate for each precursor was determined in a liquid scintillation counter and the values (cpm/mg dried eggs) were corrected for either the fertilization rate (97 %) or the activation rate (75 %).

during the third cleavage, i.e. well before the formation of the micromeres.

DNA synthesis in parthenogenetically activated eggs proceeds continuously without detectable steps. In accordance with the rate found for fertilized eggs the rate of RNA synthesis is very low at the beginning of parthenogenetic development. Its slight increase starts between 5 and 6 h p.a. but the values are still smaller than those observed in fertilized eggs.

### Rate of thymidine and uridine uptake

In the previous chapter we compared DNA and RNA synthesis in fertilized and artificially activated eggs by comparing the rate of incorporation of specific precursors. Most important for such a comparison is the

condition that in both fertilized and activated eggs the rate of uptake for these labelled precursors is identical. If this were not the case both egg samples would have a pool of precursors, each one with a different labelled proportion. It was therefore necessary to determine the rate of thymidine and uridine uptake for artificially activated and fertilized eggs.

Eggs of one female were divided into equal portions (A–E) which were processed as follows:

| Portion          | Pretreatment                        | Uptake measurements in |
|------------------|-------------------------------------|------------------------|
| A Fertilization  | Insemination                        | sea water              |
| A' Fertilization | Insemination                        | KCl solution           |
| B Artif. activ.  | thymol (40 sec)                     | sea water              |
| C Artif. activ.  | thymol (40 sec); sea water (20 min) | KCl solution           |
| D Artif. activ.  | thymol (40 sec); sea water (20 min) | sea water              |
|                  | KCl solution (70 min).              |                        |
| E Artif. activ.  | —                                   | KCl solution           |

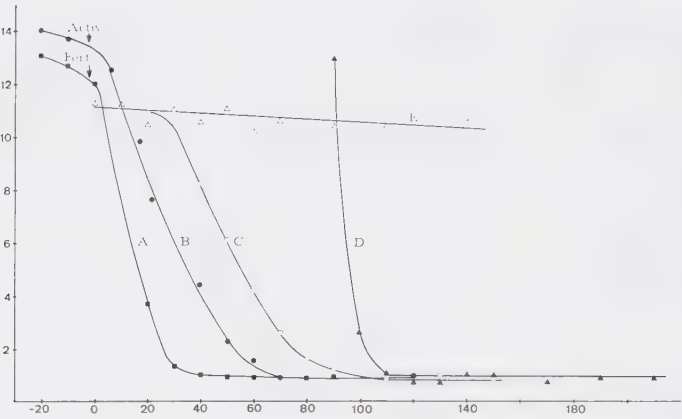
The results are summarized in fig. 12. In unfertilized eggs (beginning of curves A and B) the amount of  $^3\text{H}$ -TdR in the sea water remains practically unchanged. Upon fertilization (A) or activation with thymol (B), however, uptake rates increase markedly. The curve for thymol-activated eggs (B) has a shape similar to that obtained from fertilized eggs (A), although the uptake is somewhat slower. It reaches the plateau 30 min later than that of fertilized eggs. The decrease in radioactivity expressed by curve C is as fast as for curve B, indicating that the rate of uptake is identical. Eggs from portion D show an even faster precursor uptake than fertilized eggs. All curves end up on the same plateau. At this value, i.e. as soon as the proportion between the radioactive material which remained outside the egg and that taken up is approx. 1:13, further uptake of  $^3\text{H}$ -TdR stops. It can be concluded that the thymidine pool of both fertilized and activated eggs contains identical relative amounts

of labelled thymidine. Consequently the observed rates of incorporation of  $^3\text{H}$ -TdR into DNA give a reliable and comparable value of the rate of DNA synthesis at any given time.

In eggs of portion E finally, the precursor uptake is as slow as in unfertilized eggs.

A second experiment was performed in the same way with the modification that the supernatant was doubly labelled with  $^3\text{H}$ -UdR and  $^{14}\text{C}$ -TdR and that an additional sixth

portion of eggs (A') was tested. The uridine uptake (fig. 13a) is similar though slower than the thymidine uptake in the previous experiment. Both curves for A and for D show a similar slope. It is therefore justified to compare in the experiment of fig. 11 the RNA synthesis of fertilized and artificially activated eggs by comparing the incorporation of labelled uridine. The thymidine uptake in the double labelling experiment (fig. 13b) is distinctly slower than the uptake of thymidine alone (fig. 12). This phenomenon may be related to a competition between uridine and thymidine within a possible transport mechanism. Again, portions A and D show similar thymidine uptake which allows us to compare their DNA synthesis by measuring the incorporation of  $^{14}\text{C}$ -TdR (fig. 11). Both for  $^3\text{H}$ -UdR and even more marked for  $^{14}\text{C}$ -TdR uptake, KCl added to fertilized eggs diminished the uptake of these substances (curve A' in fig. 13a, b). All curves again reach the same plateau.



In the experiment shown in fig. 14 eggs were activated with thymol and transferred into  $^3\text{H}$ -TdR containing sea water where uptake was measured. After 20 min the same eggs were transferred into a  $^3\text{H}$ -TdR-containing KCl solution. Uptake was meas-

ured to the point where the curve reached the plateau. When the same eggs whose uptake had come to a standstill are transferred to  $^3\text{H}$ -TdR-containing sea water of the initial concentration, uptake is resumed until the same plateau is reached again. From these

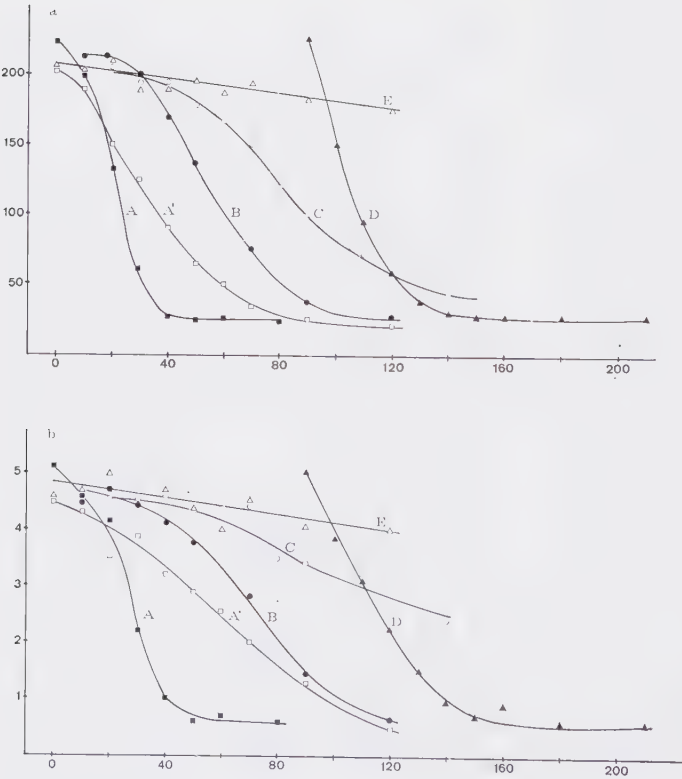


Fig. 12. Abscissa: min; ordinate:  $\times 10^3$  cpm/0.1 ml.  
Uptake of  $^3\text{H}$ -5-thymidine. Eggs, either fertilized (A) or artificially activated (B-E), were developing in labelled sea water or KCl-sea water and the decrease in radioactivity in the medium was measured. A, fertilized eggs; B, eggs in sea water immediately after activation with thymol; C, eggs in KCl-sea water previously activated with thymol and left in normal sea water for 20 min; D, eggs in sea water after a complete thymol-KCl treatment; E, eggs in KCl-sea water without any previous treatment; (further explanations in the text).

Fig. 13. Abscissa: min; ordinate:  $\times 10^3$  cpm/0.1 ml.  
Uptake of  $^3\text{H}$ -5-uridine (fig. 13a) and  $^{14}\text{C}$ -TdR (fig. 13b) from doubly labelled sea water or KCl-sea water. Measurements of the decrease in radioactivity of the supernatant. A-E, see fig. 12, A' = fertilized eggs in KCl-sea water.

data we must conclude that in those experiments in which activated eggs were transferred from one radioactive solution into another, the results are to be interpreted with caution because the pool of labelled precursor is larger in these eggs than in fertilized eggs. As incorporation is proportional to the amount of labelled precursor in the pool (unpublished results) this means that the values of DNA synthesis for parthenogenetic eggs are too high compared with fertilized eggs in those cases where transfer of eggs from one radioactive solution into another was involved. In all these experiments the eggs which were activated only with KCl proved to be nearly impermeable to thymidine and uridine (curve E in fig. 12, 13a, b). On the other hand the same eggs were able to develop and led to 10–20 % of healthy blastulae in many cases. In order to know whether in this case development of the sea urchin egg is possible without an increase of the permeability of the cell membrane we studied the rate of uptake in KCl-activated eggs after they had been transferred into sea water.

Fig. 14

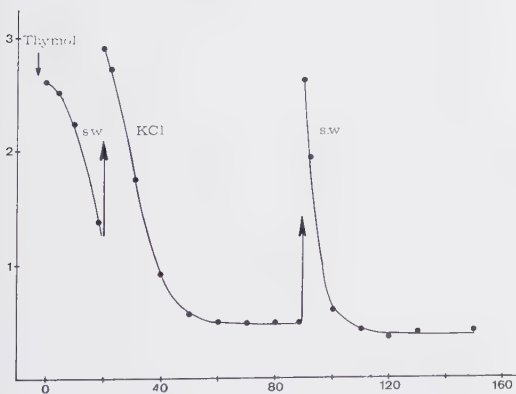


Fig. 14. Abscissa: min; ordinate:  $\times 10^3$  cpm/0.2 ml. Uptake of  $^3\text{H}$ -TdR by eggs activated with thymol-KCl. The same eggs were transferred from  $^3\text{H}$ -TdR containing sea water into a  $^3\text{H}$ -TdR containing KCl solution and again transferred into  $^3\text{H}$ -TdR-containing sea water. In each medium the decrease in radioactivity was measured.

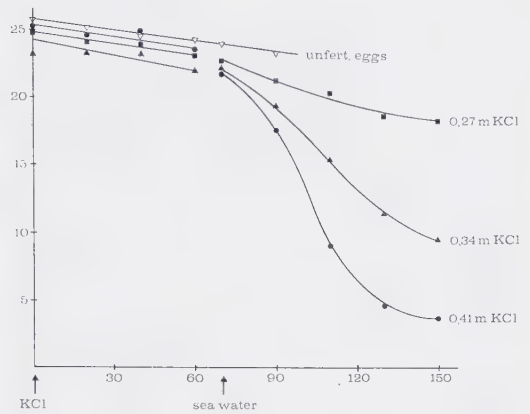


Fig. 15. Abscissa: min; ordinate:  $\times 10^3$  cpm/0.2 ml.

Uptake of  $^3\text{H}$ -TdR. Measurements of the decrease of radioactivity in the surrounding medium. Eggs were activated in 3 different concentrations of KCl in sea water containing  $^3\text{H}$ -TdR. At 70 min p.a. these three portions were transferred into  $^3\text{H}$ -TdR-containing sea water.

Eggs from one female were separated into three portions. Each portion was brought into  $^3\text{H}$ -TdR-containing sea water whose KCl concentration was either 0.27 M, 0.34 M or 0.41 M. Every 10 min, samples of the supernatant were taken. During the KCl treatment, eggs of all three KCl concentrations exhibited the same slow uptake which is typical of unfertilized eggs (fig. 15). At 70 min the eggs were transferred into  $^3\text{H}$ -TdR-containing sea water which had the same radioactivity as the KCl solution; the thymidine uptake increases markedly. This increase is proportional to the corresponding KCl concentration in which the eggs had been activated. Thus, the low uptake rate lasts only during treatment with the hyper-tonic KCl solution and increases after transfer of the eggs into sea water.

## DISCUSSION

Electronmicroscopic studies have shown that artificially activated sea urchin eggs contain centrioles similar in structure and electron

density to those present in fertilized eggs. Dirksen [16] found centrioles in the centres of cytasters and Sachs & Anderson [58] observed 1, 2 or 3 centrioles in artificially activated eggs prior to the first nuclear division. On the other hand centrioles were never found in ripe sea urchin eggs [58]. Therefore there has been speculation (see [68] and [9] for reviews) as to whether the centres of artificially induced asters derive from the egg's centriole or whether they are formed 'de novo'. Sachs & Anderson [58] suggest that the egg centriole persists in the form of submicroscopic precursors while Harvey [26] who activated non-nucleated fragments of sea urchin eggs claims that the centrioles formed under these conditions arise de novo. Morgan [48, 49] also presumes that in artificially activated sea urchin eggs the centrioles are formed 'de novo'. In our study the artificially activated sea urchin eggs collected at the beginning of the spawning season featured asters and spindles; 30 to 40 % of these eggs reached the stage of the swimming blastula. Activated eggs from the end of the spawning season rarely underwent cleavage and only 1 to 3 % developed into blastulae. This suggests that the pathway along which an egg develops depends on the degree of maturity of the egg. Considering the fact that sea urchin eggs complete meiosis before fertilization or activation, one possible explanation would be that a successful cleavage depends upon the 'age' of the egg centriole. We presume that the egg centriole which is normally suppressed by the presence of a sperm centriole can be reactivated by parthenogenetic treatment. This reactivation seems to occur more easily if the time interval between the second meiotic division and the activation is not too long. This would be true for centrioles giving rise to normal cleavage figures in the haploid mitosis as well as in cleavage following replication of

the chromosomes in a monaster. It does not, however, exclude the possibility that the centrioles, which according to Dirksen [16], are located in the centre of the numerous cytasters, are of another origin and possibly of another type.

In contrast to this season-dependent functioning of the centriole we could not detect any such seasonal variations as to the incorporation of  $^3\text{H}$ -TdR into the DNA of activated eggs. This suggests that DNA synthesis is initiated independently of the duplication of the centriole and the formation of the mitotic apparatus. Using a quite different experimental approach Bucher & Mazia [10] came to the same conclusion in the case of fertilized eggs of *Strongylocentrotus purpuratus* whose duplication of centrioles and mitosis was blocked by addition of *b*-mercaptoethanol to the sea water without DNA synthesis having been blocked.

Our radioautographs of fertilized controls did not reveal  $^3\text{H}$ -TdR incorporation into the male and female pronuclei prior to fusion. This is also true for *Arbacia punctulata* [70] and for *S. purpuratus* [10]. On the other hand Simmel & Karnofsky [60] found that in the sand dollar *Echinarachnius parma* DNA replication occurs prior to karyogamy. This was also shown to be true for rodents [2, 54], *Sabellaria* [14] and for the grasshopper *Melanophus differentialis* [65]. In contrast to our results, Anderson [3] found in electron-microscopic radioautographs that the pronuclei of *Paracentrotus lividus* incorporate  $^3\text{H}$ -TdR prior to karyogamy. He stated that, in contrast to later stages, the pronuclei lost  $^3\text{H}$ -TdR when treated with TCA. This may explain why we could not detect any label in the pronuclei prior to fusion.

Our measurements of  $^3\text{H}$ -TdR incorporation into DNA of fertilized eggs after fusion of the pronuclei are in good agreement with the results of Hinegardner et al. [31] for *S.*

*purpuratus* and with the findings of Czihak & Pohl [12] for *Paracentrotus lividus*. These authors already emphasized that as far as the four-cell stage each S phase incorporates more  $^3\text{H}$ -TdR than the preceding one. Our uptake measurements show that at least the difference in  $^3\text{H}$ -TdR incorporation between the first and the second S phase can be explained by the difference in the size of the  $^3\text{H}$ -TdR pool. This does not, however, hold true for the increasing incorporation of labelled precursor in the third S phase, since after 40 min no more thymidine is taken up and the pool remains fairly constant. (The labelled precursor incorporated in one S phase makes up only a small portion of the precursor present in the pool; unpublished results.) It seems, therefore, that  $^3\text{H}$ -TdR is not available for incorporation immediately after its uptake.

Uto & Sugiyama [66] studied the incorporation of  $^3\text{H}$ -TdR into the DNA of artificially activated eggs of *Pseudocentrotus depressus*, *Anthodiaris crassispina* and *Temnopleurus toreumaticus* by means of radioautography. In agreement with our results they found an increase in DNA synthesis at the stage of monaster formation.

There has been much discussion as to whether or not there is RNA synthesis during the early cleavage stages of sea urchins (see [13, 25, 51] for reviews). New convincing evidence has been presented recently [39, 57] demonstrating that very shortly after fertilization a complex family of RNA species is synthesized. Our results show a low incorporation rate for uridine during the first two cleavages of fertilized eggs. During the third cleavage, however, uridine incorporation increases markedly. In contrast to this, Wilt [69] found in *S. purpuratus* that uridine incorporation into an acid-insoluble fraction can not be detected before the 16-cell stage. The onset of marked RNA synthesis seems to

differ in these two sea urchin species. Baltus et al. [4] stated that artificial activation of sea urchin eggs accelerates the incorporation of  $^3\text{H}$ -UdR to the same extent as does fertilization. In our experiments, too, incorporation of  $^3\text{H}$ -UdR into activated eggs is similar though somewhat lower than in fertilized eggs, although only half the amount of nuclear DNA templates are available for RNA transcription. Since parthenogenetically activated anucleate egg fragments of *Arbacia punctulata* incorporate  $^3\text{H}$ -UdR to a similar extent as do normal embryos [11], it has been suggested that most of the RNA synthesis in the very early embryo occurs in the cytoplasm.

Our cytological results from early development confirm the findings of cytologists early in this century [30, 48, 67] and the more elaborate observations of Paspaleff [53] who had already observed the different pathways along which parthenogenetically activated sea urchin eggs may develop. In all cases where in these earlier studies the number of chromosomes in the artificially activated eggs was determined, the haploid number was found to occur, but these observations were confined to the first few divisions [15, 67]. In *Strongylocentrotus purpuratus* Hindle [30] has shown that the haploid number of chromosomes persists up to the stage of the hatched blastula. The same was found for *Mactra* [40, 41] and for *Thalassema* [42]. On the contrary, Nebel et al. [50] found a restitution of the diploid state in *Arbacia punctulata* eggs, stimulated by monochromatic ultraviolet radiation.

Our measurements showed that parthenogenetic blastulae containing either haploid, diploid or tetraploid nuclei are all fully viable. It seems that development within these limits is independent of the level of ploidy of the nuclei. The same is also true for blastulae which constitute a mosaic as to the ploidy

of their nuclei. Measurements of nuclear diameters made by Driesch [17] suggest that these different types of nuclei are also found in parthenogenetic gastrulae; he also found a few mosaic gastrulae.

Among species which show natural parthenogenesis, polyploidy is exceptionally common (see [63] for review). According to Fankhauser [20] in some amphibian species spontaneous polyploids arise from the accidental formation of unreduced gametes. In axolotl, polyploid individuals have even been raised to sexual maturity [38]. Haploid embryos also occur spontaneously; probably they owe their origin to a process of partial fertilization. Thus, also in amphibians whole sets of chromosomes may be multiplied without disturbing the harmony of gene action in development. Mosaics, too, are not exceptional in other species. In amphibians occasional  $n/2n$ ,  $n/3n$  or  $2n/3n$  mosaics have been reported (see [19] for a summary). Even mosaic mouse embryos have been observed ([18, 21] and review by [6]).

One peculiarity of the cytology of artificially activated sea urchin eggs is the formation of an intranuclear spindle (see also [53]). In some insect species, e.g. in *Acroschimus* [32] or in many of the coccids [33] chromosome replication in an intranuclear spindle is part of the normal development (see [64] for review).

In the unfertilized egg almost no uptake of thymidine or uridine was detected, but in the artificially activated egg or in the fertilized egg a rapid uptake of these precursors could be shown. The same is true for water [44], non-electrolytes [62],  $^{32}\text{P}$  [1, 35, 43], uridine in *S. purpuratus* [55], various amino acids [22], valine and cytidine [47], methionine and leucine [23] and for leucine and thymidine in *S. purpuratus* [7, 56]. Where artificially activated eggs were examined [22, 35, 43] it was found that, in accordance with our

results, artificial activation leads to a similar pattern and magnitude of uptake as does insemination. As to the mechanism of uptake, Piatigorsky & Whiteley [55] stated that in *S. purpuratus*, uridine uptake occurs against a concentration gradient, has some dependence on metabolic energy and is apparently related to activation or synthesis of a surface located enzyme. They suggest that fertilized eggs accumulate uridine by phosphorylating the nucleoside at the surface of the cell. For valine and cytidine uptake in *Paracentrotus lividus* Mitchison & Cummins [47] postulate a transport by specific carrier molecules at the cell surface. Both precursors studied by these authors showed an uptake inhibited by competitors, an apparent concentration within the cell and no back exchange. Onishi's [52] experiments on *Hemicentrotus pulcherrimus* and *Anthocardia crassispina* with ouabain (special inhibitor for ATPase) also suggest the presence of an active, ATP-dependent transport system in the oolemma.

Experiments are in progress to test the possibility of an active transport for thymidine in eggs of *Paracentrotus lividus*. It would be of especial interest to ascertain whether the permeability change following fertilization or artificial activation which results in an increased uptake for such diverse compounds as phosphates, nucleosides and amino acids is caused by a generalized change in the cell membrane, or whether these changes result from the individual activation or synthesis of transporting enzymes specific for these compounds [71].

Parthenogenetic activation seems not to be entirely equivalent to the action of the sperm: the time necessary for chromosome replication is 2.5 times longer in artificially activated eggs than in fertilized eggs, although only the haploid set has to be replicated. The same delay holds true for the beginning of marked DNA synthesis. In contrast, the

onset and rate of RNA synthesis is similar in activated and in fertilized eggs during the first few hours. According to Hultin [34] the same holds true for protein synthesis. Also regarding the thymidine and uridine uptake, the parthenogenetic agents have a similar effect as has the sperm.

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